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Cholesterol-induced hepatic inflammation does not contribute to the development of insulin resistance in male LDL receptor knockout mice



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ABSTRACT

Objective: It is generally assumed that hepatic inflammation in obesity is linked to the pathogenesis of insulin resistance. Several recent studies have shed doubt on this view, which questions the causality of this association. This study focuses on Kupffer cell-mediated hepatic inflammation as a possible driver of insulin resistance in the absence and presence of obesity.

Methods: We used male mice deficient for the low-density lipoprotein receptor (*Ldlr*^{−/−}) and susceptible to cholesterol-induced hepatic inflammation. Whole body and hepatic insulin resistance was measured in mice fed 4 diets for 2 and 15 weeks, i.e., chow, high-fat (HF), HF-cholesterol (HFC; 0.2% cholesterol) and HF without cholesterol (HFNC). Biochemical parameters in plasma and liver were measured and inflammation was determined using immunohistochemistry and RT-PCR.

Results: At 2 weeks, we did not find significant metabolic effects in either diet group, except for the mice fed a HFC diet which showed pronounced hepatic inflammation ($p < 0.05$) but normal insulin sensitivity. At 15 weeks, a significant increase in insulin levels, HOMA-IR, and hepatic insulin resistance was observed in mice fed a HFC, HFNC, and HF diet compared to chow-fed mice ($p < 0.05$). Regardless of the level of hepatic inflammation (HFC > HF, HFNC; $p < 0.05$) insulin resistance in mice fed HFC was no worse compared to mice on a HFNC and HF diet.

Conclusion: These data show that cholesterol-induced hepatic inflammation does not contribute to the development of insulin resistance in male *Ldlr*^{−/−} mice. This study suggests that Kupffer cell-driven hepatic inflammation is a consequence, not a cause, of metabolic dysfunction in obesity.

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1. Introduction

Chronic inflammation, particularly when it occurs in metabolically important organs such as the liver and adipose tissue, is considered to play a crucial role in the etiology of many metabolic diseases, including type 2 diabetes, non-alcoholic fatty liver disease (NAFLD) and cardiovascular disease [1,2]. As in adipose tissue, obesity leads to an increase in pro-inflammatory gene expression in the liver [3]. Pro-inflammatory pathways in Kupffer cells are activated in obesity and the production of inflammatory cytokines

secreted by these liver macrophages is linked to disruption of hepatic insulin signaling and reduced insulin sensitivity in mice [2–4]. For instance, LIKK mice with hepatocyte specific expression of the IκB kinase β (IKKβ), an upstream kinase that activates NF-κB, a master regulator of inflammation, exhibit profound hepatic insulin resistance with moderate systemic insulin resistance [3]. In line with this, mice lacking IKKβ in hepatocytes retain liver insulin sensitivity in response to high-fat feeding, obesity or aging [5]. Although these and many other studies suggest that insulin resistance is causally linked to hepatic inflammation [3,5,6], recent studies show a disconnection between insulin resistance and hepatic inflammation [7–11]. Moreover, obesity is a confounding factor in most studies making it difficult to dissect the role of hepatic inflammation in the development of insulin resistance.

As Kupffer cells have been studied less extensively than adipose tissue macrophages in the context of obesity and insulin resistance

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[12] and Kupffer cell depletion studies have shown controversial findings [8,13–15], we aimed to study the role of Kupffer cell-driven hepatic inflammation in the development of insulin resistance in the onset and presence of obesity. We used mice deficient for the low-density lipoprotein receptor (*Ldlr*^{-/-}) [16], a humanized mouse model exhibiting many features of the metabolic syndrome when fed a western style or diabetogenic diet, including obesity, insulin resistance, dyslipidemia, inflammation and atherosclerosis [17,18]. When triggered with low levels of cholesterol (0.15–0.2%), *Ldlr*^{-/-} mice also display many features of human NAFLD, including Kupffer cell-driven hepatic inflammation [19–21]. This is in contrast to C57BL/6 mice that develop hepatic steatosis and insulin resistance without hepatic inflammation and further progression toward non-alcoholic steatohepatitis [22]. Importantly, we have previously shown that dietary cholesterol can provoke hepatic inflammation within 7 days, in the absence of obesity [21], thereby serving as an attractive model to unravel the role of Kupffer cell-driven hepatic inflammation in the development of insulin resistance.

2. Materials and methods

2.1. Mice and diet intervention

All procedures were performed with approval of the University of Groningen Ethical Committee for Animal Experiments, which adheres to the principles and guidelines established by the European Convention for the Protection of Laboratory Animals. Experiments were carried out on male *Ldlr*^{-/-} mice (Jackson Laboratory, Bar Harbor, USA, ME), housed individually in a temperature- and light-controlled facility with *ad libitum* access to food and water.

At the age of 8–10 weeks, mice were divided into 4 groups of 8–8 mice: (1) mice fed chow diet (2181, RMH-B, Hope Farms, Woerden, the Netherlands); (2) mice fed a high-fat-diet containing 36% (w/w) fat from lard and 0.03% (w/w) cholesterol (HF; diet 4031.45, Abdiets, Woerden, the Netherlands.); (3) mice fed HF-diet containing 21% milk butter and 0.2% cholesterol (HFC; diet 0035, Scientific Animal Food and Engineering, Villemoisson-sur-orge, France); and (4) mice fed HF-diet containing 21% milk butter and 0.05% residual cholesterol derived from the butter component (HFNC; diet 0136, Scientific Animal Food and Engineering, Villemoisson-sur-orge, France). Mice were kept on these diets for 2 and 15 weeks and experiments were carried out simultaneously using diets from the same batch number. After 2 and 15 weeks animals were killed for the collection of blood, liver and muscle samples.

2.2. Dual-energy X-ray absorptiometry (DEXA) scan analysis

Fat and lean mass was determined in the same mice at 2 and 15 weeks following dietary intervention using Dual-Energy X-ray Absorptiometry (p-DEXA, Norland Stratec Medizintechnik GmbH, Birkenfeld, Germany). Mice were scanned under fed conditions while anesthetized using isoflurane and data was analyzed according to the manufacturer's instructions.

2.3. Oral glucose tolerance test

Mice were fasted for 9 h and a glucose bolus (2 g/kg of 20% glucose solution) was given by oral gavage. Glucose levels were measured with an OneTouch Ultra glucometer (Lifescan Benelux, Beerse, Belgium) before and 15, 30, 60, 90, and 120 min after the gavage.

2.4. Insulin signaling studies in vivo

The mice were fasted overnight and subjected to an intraperitoneal injection with saline or human recombinant insulin (0.75 U/kg body weight, Actrapid, Novo Nordisk Canada inc., Ontario, Canada) 15 min before killing. Tissues were isolated and snap-frozen in liquid nitrogen.

2.5. RT-PCR

Total RNA was isolated from the liver with TRIzol reagent (Sigma Aldrich, Zwijndrecht, the Netherlands) and total RNA (1 µg) from each individual mouse was converted into cDNA with Quantitect Reverse Transcription kit (QIAGEN, Venlo, the Netherlands). Real time PCR (RT-PCR) was performed using a 7900HT system (Applied Biosystems, Warrington, UK) and values were corrected using the housekeeping gene Cyclophilin A (*Ppia*). The following primers were used: *F4/80* forward TGTGTCGTGCTGTTCAAGACC, reverse AGGAATCCCGCAATGATGG; *Clec4f* forward TGAGTGGAA-TAAAGAGCTCCC, reverse TCATAGTCCCTAAGCCTCTGGA; *Mcp-1* forward GCTGGAGAGCTACAAGAGGATCA, reverse ACAGACCTCTCTCTTGTAGCTTGGT; *Cmklr1*, forward AACAGCCACTACCA-GAACAAC, reverse CGTGACTGGAAATACCTTCTC; *Tnf-α*, forward CATCTTCTCAAAATTCGAGTGACAA, reverse TGGGAGTAGA-CAAGGTACAACCC and *Il-1β*, forward TGCAGCTGGAGAGTGTTGG, reverse TGCTTGTGAGGTGCTGATG; *Ppia* forward TTCCTCTTTCA-CAGAATTATTCCA, reverse CCGCCAGTGCCATTATGG.

2.6. Immunoblot analysis

Tissues were homogenized in ice-cold buffer (liver: NaCl 150 mM, Tris-HCl pH 7.5 50 mM, EDTA 5 mM, Pyrophosphate 30 mM, NaF 50 mM, Triton X-100, PMSF 100 mM, phosphatase inhibitor cocktails; skeletal muscle: as shown previously [23]). Equal amounts of protein were separated by SDS-PAGE, and transferred to Polyvinylidene Difluoride membranes (GE Healthcare Life Sciences, Diegem, Belgium). Phosphorylated and total AKT antibodies were purchased from Cell Signaling Technology (Leiden, the Netherlands). Immune-complexes were visualized by chemiluminescence (GE Healthcare Life Sciences, Diegem, Belgium) and quantified by densitometry (imageJ software, National Institute of Health).

2.7. Statistical analysis

Data are expressed as means ± SEM for the indicated number of observations. Statistical significance between groups was determined using a two-tailed Mann–Whitney *U* test. Multiple comparison analysis was performed using two-way ANOVA with a Bonferroni post hoc test to correct for multiple testing. Two-way ANOVA for repeated measurements was used to test for comparisons in body weight and fat mass between the 2- and 15-week time points. Values of *p* < 0.05 were considered statistically significant. The software used for the analysis was GraphPad Prism (version 5.00 for Windows, GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Dietary cholesterol triggers Kupffer cell activation

To confirm Kupffer cell involvement following cholesterol supplementation to the diet, immunostaining against Cd68 and Cd11b was performed in liver sections of *Ldlr*^{-/-} mice fed chow, HF, HFC and HFNC diet for 2 and 15 weeks. Indeed, macrophage size and number (Fig. 1A) was significantly increased in the livers of 2-week

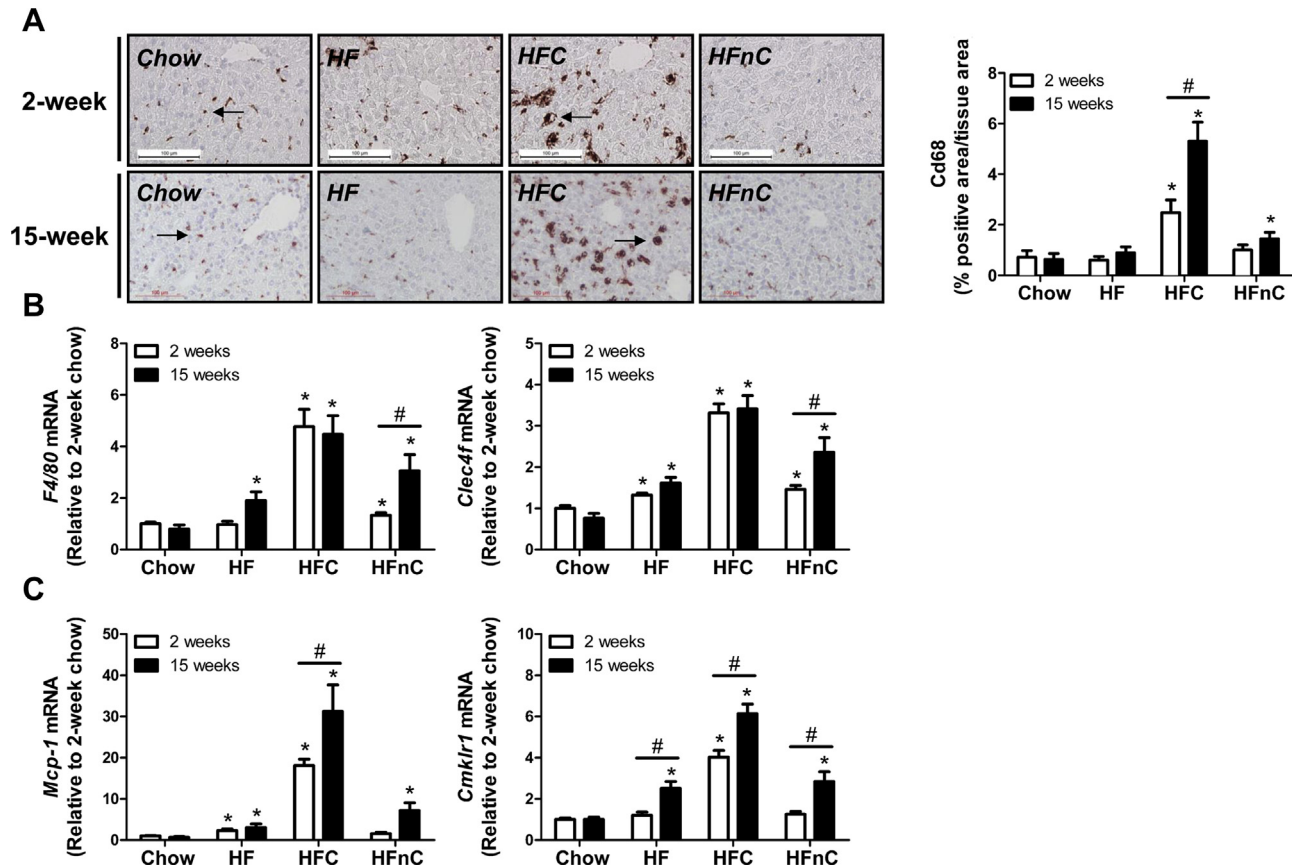


Fig. 1. Dietary cholesterol triggers Kupffer Cell Activation. (A) Representative pictures of Cd68 staining and morphometric analysis of Cd68 stained liver sections from *Ldlr*^{-/-} mice fed a chow, HF, HFC or HFNC diet for 2 and 15 weeks. Original magnification $\times 200$. Kupffer cells are indicated with arrows. (B) RT-PCR analysis of hepatic *F4/80* and *Clec4f* and (C) *Mcp-1* and *Cmk1r1* mRNA expression. Data is expressed as fold induction vs 2-week chow-fed *Ldlr*^{-/-} mice. Data are expressed as means \pm SEM, $n = 6$ in each group. * $p < 0.05$ vs chow-fed mice (Mann–Whitney *U* Test); # $p < 0.05$ 2- vs 15-week diet period (two-way ANOVA).

HFC-fed mice when compared to mice fed chow, HF and HFNC diet ($p < 0.05$). Consistent with this, the number of Cd11b⁺ macrophages was significantly increased in *Ldlr*^{-/-} mice after 2 weeks of HFC feeding when compared to mice fed chow, HF and HFNC diet (Supplemental Fig. 1; $p < 0.05$). In addition, 2 weeks of HFC feeding resulted in an increase in the expression levels of the specific Kupffer cell markers *F4/80* (Fig. 1B; $p < 0.05$ vs chow, HF, HFNC) and *Clec4f* (C-type lectin domain family 4, member f; Fig. 1B; $p < 0.05$ vs chow, HF, HFNC). Consistent with this, HFC feeding resulted in a concomitant increase in the expression level of monocyte chemotactic protein-1 (*Mcp-1*, Fig. 1C) and chemokine-like receptor 1 (*Cmk1r1*, Fig. 1C; $p < 0.05$ vs chow, HF, HFNC, ANOVA), genes known to be involved in monocyte/macrophage migration and infiltration [12,24].

Similar to 2 weeks of HFC feeding, feeding mice a HFC diet for 15 weeks resulted in a marked increase in Kupffer cell size and number (Fig. 1A), and Cd11b cell count (Supplemental Fig. 1) when compared to mice fed chow, HF and HFNC diet for 15 weeks ($p < 0.05$, ANOVA). In addition, Kupffer cell number was significantly enhanced in liver sections of *Ldlr*^{-/-} mice subjected to 15 weeks of HFC feeding compared to 2 weeks of HFC feeding (Fig. 1A). However, the number of Cd11b⁺ macrophages in liver sections of *Ldlr*^{-/-} mice fed a HFC diet did not change overtime (Supplemental Fig. 1). In addition, hepatic *F4/80* and *Clec4f* expression levels were not significantly increased in 15-week HFC-fed mice compared to 2-weeks HFC-fed mice (Fig. 1B; ANOVA). Furthermore, hepatic *Mcp-1* and *Cmk1r1* levels were increased in 15-week HFC-fed mice compared to 2-week HFC-fed mice (Fig. 1C). 15 weeks of high-fat feeding with low levels

of cholesterol (HF, HFNC) also led to a significant increase in *F4/80*, *Clec4f*, *Mcp-1* and *Cmk1r1* expression compared to chow-fed *Ldlr*^{-/-} mice (Fig. 1B, C). However, this increase was relatively small compared to mice fed a HFC diet for 15 weeks (*F4/80*, HF vs HFC $p < 0.01$; HFNC vs HFC $p < 0.2468$; *Clec4f*, HF vs HFC $p < 0.01$; HFNC vs HFC $p < 0.1320$; *Mcp-1*, HF vs HFC $p < 0.01$; HFNC vs HFC $p < 0.01$; *Cmk1r1*, HF vs HFC $p < 0.01$; HFNC vs HFC $p < 0.01$).

3.2. Hepatic inflammation is sustained after long-term HFC feeding in *Ldlr*^{-/-} mice

To assess whether enhanced Kupffer cell activation translates into increased hepatic inflammation, we performed gene expression analysis for the inflammatory mediators *Tnf- α* , and *Il-1 β* in the livers of *Ldlr*^{-/-} mice at both time points. The expression of these genes was significantly increased in *Ldlr*^{-/-} mice fed a HFC diet for 2 weeks when compared to mice fed chow, HF and HFNC diet (Fig. 2A, B, $p < 0.05$). Mice fed a HF diet containing 0.03% cholesterol also showed a significant up regulation of these genes above chow levels (Fig. 2A, B), but this was only modest compared to HFC-fed mice (*Tnf- α* , HF vs HFC $p < 0.01$; *Il-1 β* , HF vs HFC $p < 0.01$). Moreover, hepatic inflammation was absent in the mice fed a HFNC diet for 2 weeks, highlighting the specificity of our cholesterol supplementation model in inducing hepatic inflammation.

In addition, 15 weeks of HFC feeding led to a similar significant increase in inflammatory gene expression compared to 2 weeks of HFC feeding (Fig. 2A, B). However, it did not further increase the levels of these genes above the 2-week time point.

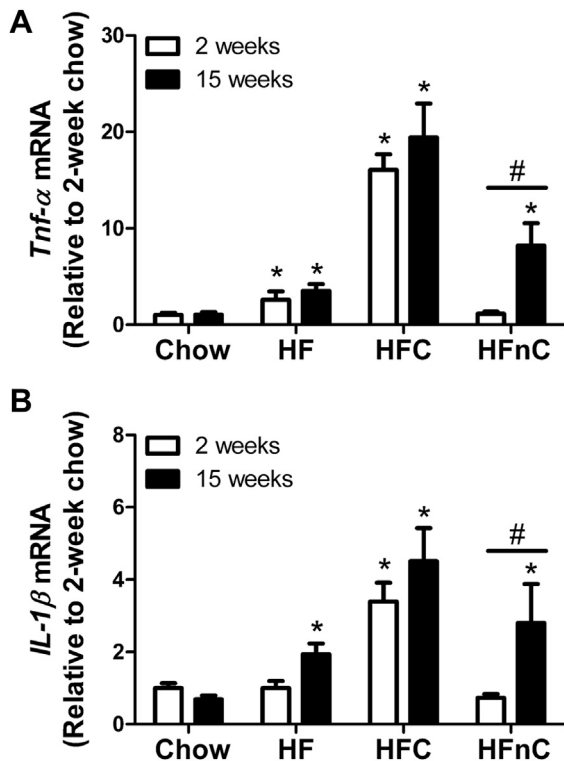


Fig. 2. Kupffer cell-mediated hepatic inflammation is sustained after long-term HFC feeding in *Ldlr*^{-/-} mice. (A) RT-PCR analysis of *Tnf-α* and (B) *IL-1β* mRNA expression in livers from *Ldlr*^{-/-} mice fed a chow, HF, HFC or HFNC diet for 2 and 15 weeks. Data is expressed as fold induction vs 2-week chow-fed *Ldlr*^{-/-} mice. Data are expressed as means ± SEM, $n = 6$ in each group. * $p < 0.05$ vs chow-fed mice (Mann–Whitney *U* Test); # $p < 0.05$ 2- vs 15-week diet period (two-way ANOVA).

15 weeks of HF-feeding also led to significant increase in hepatic inflammation, albeit less pronounced compared to HFC feeding (*Tnf-α*, HF vs HFC $p < 0.01$; *IL-1β*, HF vs HFC $p < 0.05$). Similar results were obtained for 15 weeks of HFNC feeding in mice, although *IL-1β* did not reach statistical significance (*Tnf-α*, HFNC vs HFC $p < 0.05$).

3.3. Dietary cholesterol does not lead to overt changes in adiposity in *Ldlr*^{-/-} mice

As body weight adversely affects insulin sensitivity, *Ldlr*^{-/-} mice were subjected to DEXA scan analysis to determine fat mass at the end of the 2- and 15-week diet period. After 2 weeks, body weight (Fig. 3A) and fat mass (Fig. 3B) did not differ significantly amongst the 4 diet groups. At the end of the 15-week diet period body weight (Fig. 3A) and fat mass (Fig. 3B) were significantly increased in mice fed a HFC and HF diet compared to the 15-week chow controls and the respective 2-week time points (Fig. 3A, B). However, body weight was lower in 15-week HFC-fed mice compared to HF-fed mice ($p < 0.05$), although fat mass did not differ significantly between these groups (Fig. 3B; HFC vs HF $p = 0.20$). In addition, body weight was significantly lower in HFNC-fed mice compared to both HFC- and HF-fed mice (Fig. 3A; HFNC vs HFC $p < 0.05$; HFNC vs HF $p < 0.01$). Plasma and liver cholesterol levels were increased in *Ldlr*^{-/-} mice fed a HFC, HF and HFNC diet compared to chow-fed mice at either time point (Supplemental Tables 1, 2). In addition, plasma and liver cholesterol levels were markedly increased in HFC-fed mice compared to mice fed HFNC and HF diet. Furthermore, we observed higher plasma and hepatic TC levels in HFNC-

fed mice compared to HF-fed mice (Supplemental Tables 1, 2). This may be due to the higher residual cholesterol component in the HFNC diet (0.05%) compared to the HF diet (0.03%). Plasma triglycerides (TG) were elevated in all mice following HF feeding at 2 and 15 weeks compared to chow controls and no differences were observed between HFC-, HFNC- and HF-fed mice at both time points (Supplemental Tables 1, 2). Furthermore, hepatic TG levels were significantly increased in mice fed a HFC, HF and HFNC diet compared to chow-fed mice at the 2-week time point (Supplemental Table 1) and did not differ at the 15-week time point (Supplemental Table 2).

3.4. Hepatic inflammation does not induce insulin resistance in lean *Ldlr*^{-/-} mice

To investigate whether hepatic inflammation may affect glucose metabolism, we assessed glucose tolerance, plasma insulin levels, and the calculated HOMA-IR index as markers of insulin resistance. At 2 weeks, *Ldlr*^{-/-} mice fed a HFC diet did not exhibit elevated fasted insulin levels compared to mice fed a HF and HFNC diet (Fig. 4A) nor was their glucose tolerance negatively affected (Fig. 4B). Consistent with this, HOMA-IR was not significantly increased in the mice fed a HFC diet (Fig. 4C) compared to chow, HF-, or HFNC-fed mice, suggesting that cholesterol-induced hepatic inflammation does not induce the development of systemic insulin resistance. In addition, no differences were observed in phosphorylation status of AKT in either skeletal muscle or liver (Fig. 4D) in the *Ldlr*^{-/-} mice fed a HFC diet compared to chow-, HF-, or HFNC-fed mice, suggesting that insulin signaling in these metabolically active tissues was not impaired.

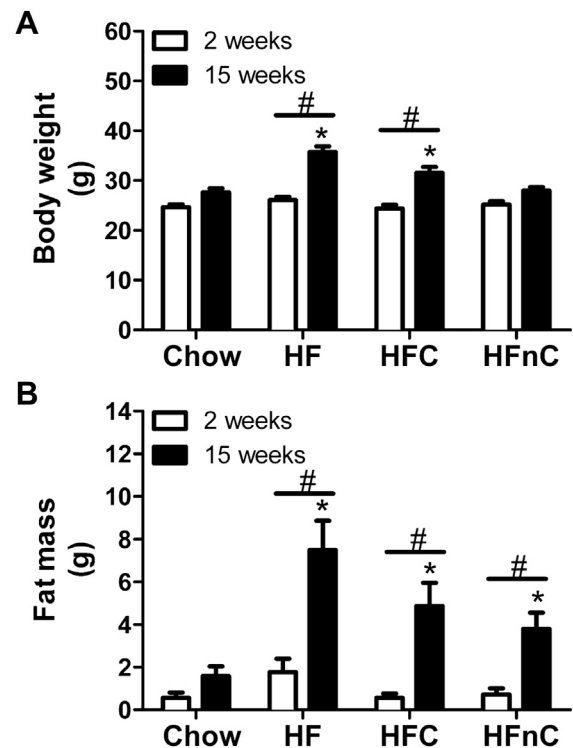


Fig. 3. Dietary cholesterol does not lead to overt changes in adiposity in *Ldlr*^{-/-} mice. (A) Body weight of *Ldlr*^{-/-} mice fed a chow, HF, HFC or HFNC diet for 2 ($n = 12$ in each group) and 15 weeks ($n = 12$ in each group). (B) Fat mass was determined by DEXA scan analysis ($n = 6$ in each group). Data are expressed as means ± SEM. * $p < 0.05$ vs chow-fed mice (Mann–Whitney *U* Test); # $p < 0.05$ 2- vs 15-week diet period (two-way ANOVA for repeated measurements).

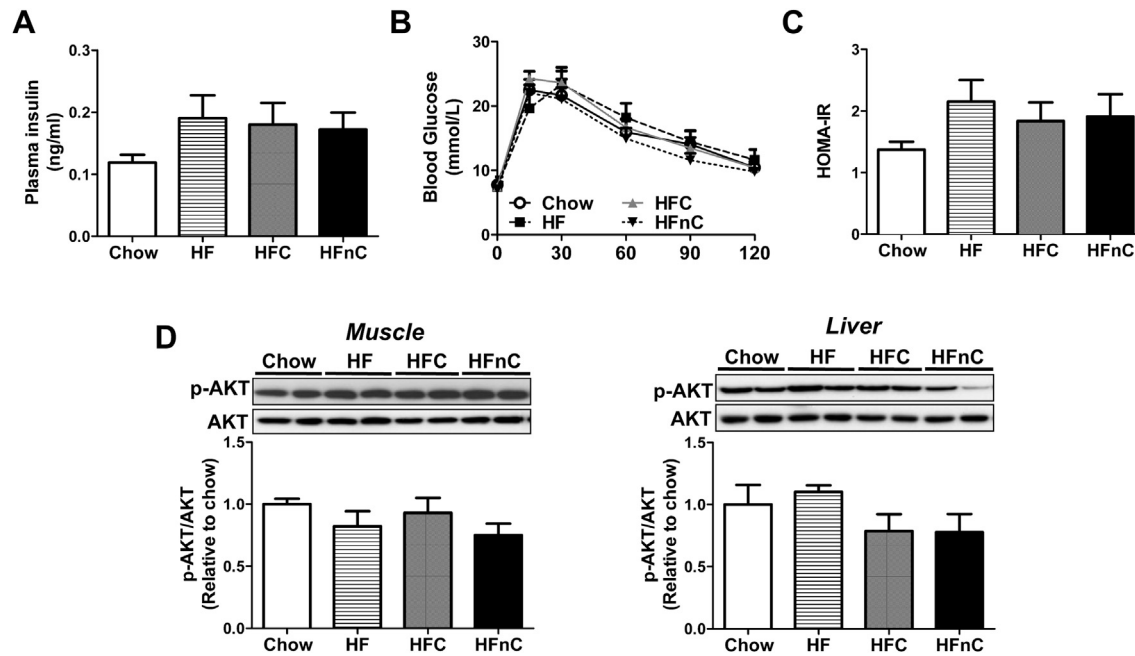


Fig. 4. Hepatic inflammation does not induce insulin resistance in lean *Ldlr*^{-/-} mice. (A) Plasma insulin levels in *Ldlr*^{-/-} mice fed a chow, HF, HFC or HFNC diet for 2 weeks ($n = 6$ in each group). (B) Blood glucose levels during an oral glucose tolerance test ($n = 6$ in each group). (C) HOMA-IR as a surrogate marker of insulin resistance ($n = 6$ in each group). (D) Western blot analysis of phosphorylated and total AKT in skeletal muscle and liver after an insulin injection ($n = 8$ in each group). * $p < 0.05$ vs chow-fed mice (Mann–Whitney U Test). Data are expressed as means \pm SEM.

3.5. Hepatic inflammation does not aggravate insulin resistance in obese *Ldlr*^{-/-} mice

Although our data indicate that Kupffer cell-mediated hepatic inflammation may not increase the susceptibility toward the development of insulin resistance during the onset of obesity, it may still aggravate insulin resistance in obese *Ldlr*^{-/-} mice. However, glucose tolerance was not affected in *Ldlr*^{-/-} mice fed either HFC or HFNC diet, and was only impaired in mice fed the diabetogenic HF diet (Fig. 5A). In spite of this, fasted insulin levels (Fig. 5B) and HOMA-IR (Fig. 5C) were all significantly increased compared to the chow controls but did not differ between the HFC-, HFNC-, and HF-fed groups. Whereas, insulin signaling in skeletal muscle did not seem to be impaired after 15 weeks of HFC-, HFNC- and HF-feeding compared to chow-fed mice, hepatic insulin resistance was observed in mice on a HFC diet but again was no worse compared to mice on a HFNC and HF diet (Fig. 5D). This was indicated by a similar reduction in insulin-stimulated AKT phosphorylation in the livers of these mice (Fig. 5D).

4. Discussion

In this study, we have explored the role of hepatic inflammation as a possible driver of insulin resistance in the onset and presence of obesity. We show that *Ldlr*^{-/-} mice with pronounced hepatic inflammation, induced by a 2-week HFC-diet, do not exhibit signs of insulin resistance in comparison to mice fed a HFNC-diet (Fig. 4). We also show that insulin resistance is not aggravated in obese *Ldlr*^{-/-} mice with sustained hepatic inflammation induced by a 15-week HFC-diet (Fig. 5) compared to mice fed a HFNC-diet. Moreover, we did not find any correlation between the level of hepatic inflammation, insulin resistance and/or obesity in these mice (Supplemental Table 3). Therefore, our data show that HFC-induced hepatic inflammation per se does not cause insulin resistance in *Ldlr*^{-/-} mice.

Although previous studies have elegantly shown that insulin resistance is causally related to hepatic inflammation [3,5], our data do not support these findings. The reason for this is unclear; however, it may be related to the experimental model used to assess the causality of this association. In this study, we used a Kupffer cell-based approach to drive hepatic inflammation, whereas hepatic inflammation in LIKK mice [3] and *Ikkb*^{Δ^{hep} mice [5] is hepatocyte-driven. This raises the question whether hepatocyte-driven inflammation is functionally different from Kupffer cell-driven inflammation and whether or not there is a distinctive role in their control of insulin resistance. Nonetheless, it should be noted that *Ikkb*^{Δ^{hep} mice retain liver insulin responsiveness in response to a high-fat diet, obesity and aging, but continue to develop peripheral insulin resistance in muscle and fat [5]. By contrast, LIKK mice expressing constitutively active (IKK β) in hepatocytes exhibit insulin resistance both locally in liver and systemically [3]. Therefore, the possibility arises that a causal relationship between hepatic inflammation and insulin resistance may only exist in the liver and not systemically. In addition, deletion of *Ikkb* in myeloid cells was reported to lead to a global improvement in insulin sensitivity in *Ikkb*^{Δ^{mye} mice [5] but this effect may not be attributed to Kupffer cells in the liver as the LysM promoter used to generate these mice is not active in Kupffer cells [25]. However, Kupffer cell depletion studies have shown conflicting results, and have both been associated with improvement [13–15] and deterioration of hepatic insulin resistance [8], thereby questioning a role of Kupffer cells in control of hepatic and global insulin sensitivity.}}}

In this study we have assessed parameters on two different scales, i.e. inflammation and insulin resistance. On these two scales, changes may not be in the same magnitude. Therefore, we cannot exclude that the absence of insulin resistance at week 2 of HFC-feeding may in fact reflect a temporal delay of onset of insulin resistance induced by hepatic inflammation. Nevertheless, several other reports have also raised doubts on the current concept that hepatic inflammation causes insulin resistance in mice. Mice lacking the TNF receptors, p55 and p75, do not show improvement of insulin resistance despite

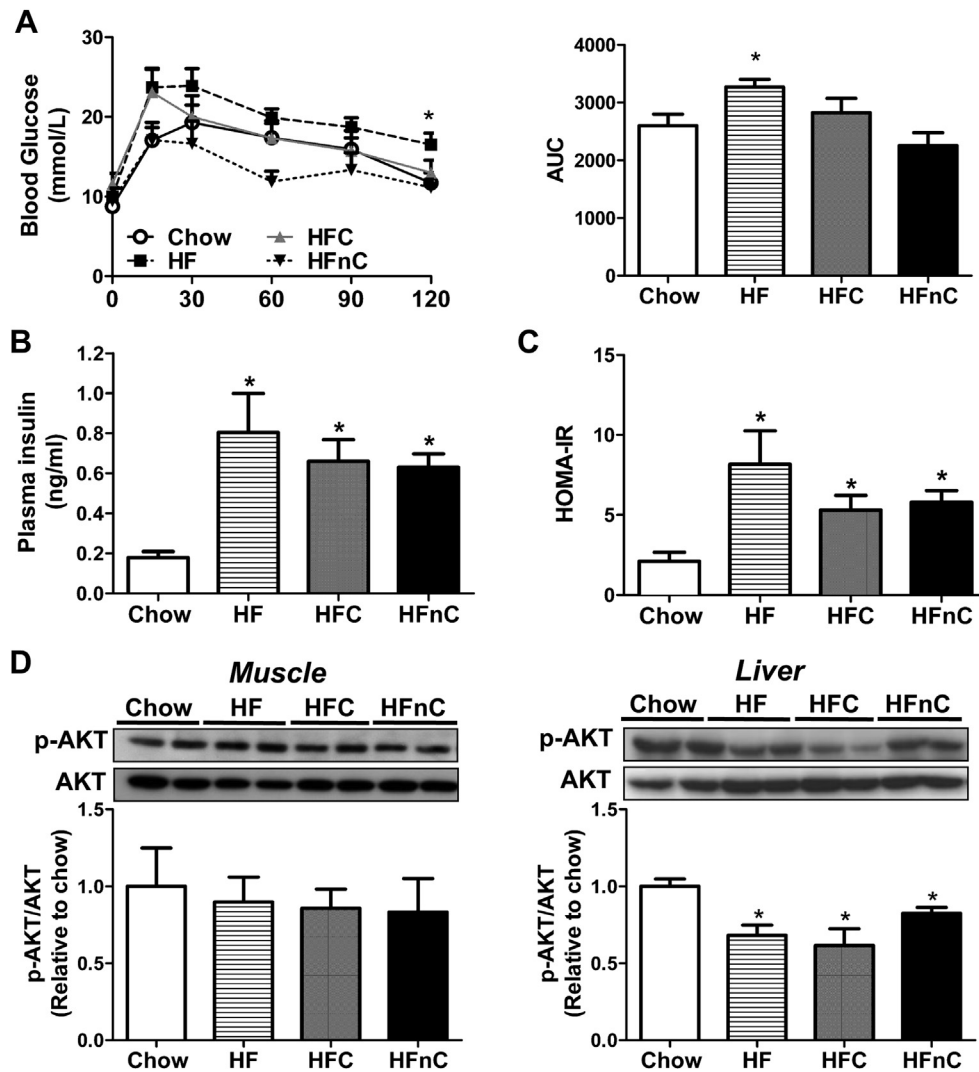


Fig. 5. Hepatic inflammation does not aggravate insulin resistance in obese *Ldlr*^{-/-} mice. (A) Blood glucose levels during an oral glucose tolerance test and AUC for the glucose tolerance test in *Ldlr*^{-/-} mice at 15 weeks on a chow, HF, HFC or HFnC diet ($n = 6$ in each group). (B) Plasma insulin levels ($n = 6$ in each group). (C) HOMA-IR ($n = 6$ in each group). (D) Representative western blot analysis of phosphorylated and total AKT in skeletal muscle and liver after an insulin injection ($n = 8$ in each group). * $p < 0.05$ vs chow-fed mice (Mann–Whitney U Test). Data are expressed as means \pm SEM.

reduced levels of hepatic inflammation [11]. Consistent with this, mice deficient for MyD88, an adapter protein for TLR/IL-1 receptor signaling, are more prone to develop metabolic disease in response to HF feeding despite lower levels of inflammation [9]. Furthermore, hepatic insulin resistance is not always associated with the presence of inflammation in the liver as *Liv*-diacylglycerol O-acyltransferase 2 (DGAT2) mice fed a chow diet show hepatic insulin resistance in the absence of liver inflammation [10]. In addition, we have recently shown that hepatic inflammation is not associated with insulin resistance in TNFR1 non-sheddable mice with a gain of function mutation in the TNFR1 resulting in chronic low-grade inflammation in the liver [7]. Moreover, mice with the non-shedding mutation fed a chow diet for one year were not prone to developing insulin resistance, nor did 12 weeks of HF feeding at the age of one year accelerate the onset of insulin resistance in these mice [7]. Therefore, our data and the above-discussed studies all show, using different experimental approaches (genetically engineered mouse models, Kupffer cell depletion, and dietary intervention), a disconnection between hepatic inflammation and the existence of insulin resistance. A similar dissociation has also been observed between hepatic steatosis and insulin resistance in various genetically and

pharmacologically manipulated mouse models [26–29] and warrants further study.

Our data suggest that other obesity-associated factors may be responsible for the development of insulin resistance in our mice. Obesity is known to promote insulin resistance and adiposity was significantly increased in all HF diet groups following 15 weeks of HF feeding (Fig. 3) whereas inflammatory gene expression in the liver did not increase above the 2-week time point (Fig. 2). Moreover, systemic and hepatic insulin resistance only became apparent with increasing adiposity in *Ldlr*^{-/-} mice suggesting that signals originating from the adipose tissue rather than the liver may have interfered with proper insulin signaling in the *Ldlr*^{-/-} mice. This is consistent with recent data showing that increased inflammation in both liver and muscle only became apparent after establishment of obesity [4]. Our studies cannot answer the question whether total adiposity may be the driving force behind the development of insulin resistance in our model or whether site-specific fat depots (visceral or subcutaneous adipose tissue), a reduced capacity to store excess nutrients, and/or an altered secretion of adipokines may be involved. In addition, it has previously been shown that dietary lipids and cholesterol (high-fat diet feeding) induce the infiltration

of macrophages into the pancreatic islets, leading to the production/secretion of inflammatory cytokines and chemokines by these infiltrated macrophages [30–32]. Consequently this leads to beta cell dysfunction and in impaired insulin secretion and hyperglycemia [30–32]. As we observed a significant increase in the insulin levels following 15-weeks of dietary intervention in all HF-diet groups compared to chow-fed feeding (Fig. 5B), our data may thus confirm a role for beta-cell involvement in the development of insulin resistance in our study. Although cholesterol may contribute to impaired beta cell function and glucose intolerance in *Ldlr*^{−/−} mice [33], HFC feeding did not aggravate the level of insulin resistance in our study thereby excluding a role for cholesterol-driven beta-cell impairment in the development of insulin resistance.

In summary, our results demonstrate that the level of Kupffer cell-mediated hepatic inflammation is not directly correlated to the development of insulin resistance in male *Ldlr*^{−/−} mice. Similar to the dissociation between hepatic steatosis and insulin resistance, we show dissociation between hepatic inflammation and the development of insulin resistance in mice. Therefore, our data provides evidence to question a possible role of Kupffer cell-driven hepatic inflammation in insulin resistance in mice. However, in order to exclude Kupffer cell-driven hepatic inflammation as a cause of metabolic dysfunction in obesity, Kupffer cell depletion studies need to be performed.

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Conflict of interest

The authors have nothing to disclose.

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Appendix A. Supplementary material

Supplementary data related to this article can be found online at <http://dx.doi.org/10.1016/j.atherosclerosis.2013.11.074>.

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